Veterinary Immunology and Immunopathology 191 (2017) 74–79

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Protection of commercial turkeys following inactivated or recombinant H5 vaccine application against the 2015 U.S. H5N2 clade 2.3.4.4 highly pathogenic avian influenza virus

Darrell R. Kapczynski[a,*,1], Matthew J. Sylte[b,1], Mary L. Killian[c], Mia K. Torchetti[c], Klaudia Chrzastek[a], David L. Suarez[a]

[a] Exotic and Emerging Avian Viral Diseases Research Unit, U.S. National Poultry Research Center, Agricultural Research Service, U.S. Department of Agriculture, 934 College Station Road, Athens, GA, 30605, United States

[b] Food Safety and Enteric Pathogens Research Unit, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, 1920 Dayton Avenue, Ames, IA, 50010, United States

[c] National Veterinary Services Laboratories, Veterinary Services, Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Ames, IA 50010, United States

ARTICLE INFO

Keywords:
Turkey (*Meleagris gallopavo*)
Highly pathogenic avian influenza virus
Vaccine efficacy
Viral shedding
Reverse genetics
Recombinant vaccine

ABSTRACT

Between December 2014 and June 2015, North America experienced the largest recorded foreign animal disease outbreak with over 47 million poultry dead or euthanized from viral exposure to a clade 2.3.4.4 H5 highly pathogenic avian influenza (HPAI) epizootic. Soon after the epizootic began, the U.S. Department of Agriculture (USDA) began testing the efficacy of different vaccines as a possible future control strategy. The aim of these studies were to evaluate the efficacy three H5 vaccines to aid in control of HPAI in commercial turkeys. Three different vaccine technologies were evaluated for efficacy: 1) inactivated reverse genetic laboratory-generated virus encoding a clade 2.3.4.4 H5 hemagglutinin (HA) gene (rgH5), 2) recombinant turkey herpesvirus encoding a clade 2.2. H5 HA (rHVT-AI), and 3) recombinant replication-deficient alphavirus RNA particle vaccine encoding a clade 2.3.4.4 H5 HA (RP-H5). All vaccines tested significantly ($P < 0.01$) increased survival rates between vaccinated and sham vaccinated groups of poults challenged with A/turkey/Minnesota/12582/2015 clade 2.3.4.4 H5N2 HPAI. The rgH5 vaccine had detectable serum hemagglutination inhibition (HI) antibody against the challenge virus, and significantly reduced the frequency and level of viral shedding from oropharyngeal and cloacal swabs at days 2 and 4 post-challenge. Vaccination with only rHVT-AI or RP-H5 was not 100% protective, and failed to significantly reduce viral shedding post-challenge. A combined prime and boost strategy with the rHVT-AI and RP-H5, or rHVT-AI and rgH5, was 100% protective against lethal H5N2 HPAI challenge.

Results of these studies led to USDA conditional approval of commercially available recombinant vaccines for use in turkeys as a control measure for clade 2.3.4.4 H5 HPAI epizootics.

1. Introduction

The 2014-15 North American clade 2.3.4.4 hemagglutinin subtype 5 (H5) highly pathogenic avian influenza (HPAI) epizootic began in November 2014 when Eurasian HPAI H5N8 was detected in wild birds (Ip et al., 2015; Lee et al., 2015; Torchetti et al., 2015) and poultry along the Pacific coast of North America (Stoute et al., 2016). Initially, Eurasian H5N8 and an H5N2 reassortment, which were poorly adapted for replication in chickens, were identified (Bertran et al., 2016). Soon after, H5N8 reassorted with other North American AI viruses to produce a HPAI H5N1 (Torchetti et al., 2015) and a HPAI H5N2, the latter of which became poultry adapted. Infections with the H5N2 HPAI devastated poultry production in the Midwest of USA, with approximately 47 million poultry euthanized or dead after exposure to the virus (Cima,

Abbreviations: APHIS, the animal and plant health inspection service; ABSL3, animal biosafety level 3; BSL3Ag, biosafety level 3 for agriculture group housed animals; BPL, beta-propiolactone; BSL3E, biosafety level 3 elevated; DIVA, differentiate infected from vaccinated animal; HA, Hemagglutinin; HAU, hemagglutinin unit; HI, hemagglutinin inhibition; HPAI, highly pathogenic avian influenza; LPAI, Low pathogenic avian influenza; NADC, National Animal Disease Center; qRRT-PCR, quantitative real time RT-PCR; rgH5, reverse genetics virus encoding H5; rHVT-AI, recombinant herpesvirus of turkey encoding H5 hemagglutinin gene; RP-H5, replication-deficient alphavirus RNA particle vaccine encoding H5; SPF, specific pathogen free; SQ, subcutaneous

* Corresponding author.
E-mail address: darrell.kapczynski@usda.ars.gov (D.R. Kapczynski).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.vetimm.2017.08.001
Received 24 March 2017; Received in revised form 25 July 2017; Accepted 3 August 2017

2015). The majority of commercial poultry affected were egg-laying chickens and turkeys, which manifested as depression, diarrhea and neurological disease (e.g., torticollis). To control the epizootic, eradication (stamping out) was used without vaccination. As of November 2015, the USA was declared free of the clade 2.3.4.4 H5N2 HPAI epizootic. The estimated economic losses from the clade 2.3.4.4 H5N2 HPAI epizootic range up to $3.3 billion USD, and resulted in 18 trade partners banning import of U.S. poultry.

In early 2015, the U.S. Department of Agriculture (USDA) began avian influenza (AI) vaccine efficacy studies to assess their value as a potential tool to control clade 2.3.4.4 H5N2 HPAI. Since 1959, there have been 37 HPAI epizootics across the world. Thirty-two of the 37 epizootics used stamping-out protocols as the sole control strategy, which led to their rapid eradication. The 5 other epizootics used vaccines and stamping-out to control the disease, with the goal that vaccination would reduce the amount of virus in poultry populations and slow or stop the flock to flock transmission of the virus. When used properly and in conjunction with other control measures, vaccines can be an effective method to control virus, and has led to successful HPAI eradication (Swayne, 2012; Swayne et al., 2014). However, previous H5N1 HPAI epizootics have occurred in vaccinated chicken flocks. This may be the result of improper administration, lack of timely seroconversion before HPAI exposure or use of poor quality vaccine. Antigenic drift in field avian influenza (AI) viruses has resulted in failure of protection by classic H5 vaccines strains in Mexico, China, Egypt, Indonesia, Hong Kong and Vietnam (Swayne et al., 2014). This problem can been met by developing new vaccines with strains that provide matched protection against an ever-changing repertoire of HPAI surface antigens. Initial studies in chickens indicated the historic USDA H5 vaccine bank strains (e.g., Tk/WI/68) produced only partial protection against clade 2.3.4.4 H5N2 HPAI, suggesting that it was time to evaluate new vaccines to combat this new clade (Kapczynski et al., 2017). Poultry vaccines are commonly tested in chickens as a model system, but because the U.S. outbreak had such a large turkey component, vaccination studies that were conducted in turkeys was thought to be critical to properly evaluate efficacy in the target population. Three different types of recombinant H5 vaccines were tested: 1) inactivated reverse genetic (rg) laboratory-generated recombinant virus encoding clade 2.3.4.4 H5 gene (rgH5), 2) recombinant turkey herpesvirus encoding clade 2.2. H5 gene (rHVT-AI), and 3) recombinant replication-deficient alphavirus RNA particle vaccine encoding clade 2.3.4.4 H5 gene (RP-H5). The H5 antigens used in vaccines included clades 2.3.4.4 or 2.2 HPAI with the HA cleavage site modified to low pathogenic avian influenza (LPAI) virus genotype. Vaccines were administered to commercial turkeys alone or in combination (prime-boost) and tested for protection against clade 2.3.4.4 H5N2 HPAI challenge.

2. Materials and methods

2.1. Viruses

The highly pathogenic A/turkey/Minnesota/12582/2015 (TK/MN/15) H5N2 clade 2.3.4.4 was used as the challenge viruses. Virus was propagated in specific pathogen free (SPF) embryonated chicken eggs according to standard procedures (Williams, 2016). Allantoic fluid containing virus was harvested for titration of HPAI challenge virus using a hemagglutination assay (Kapczynski et al., 2017). All experiments using HPAI viruses, including work with animals, were reviewed by the institutional biosecurity committee and were performed in animal biosecurity level-3 enhanced (ABSL3E) facilities at the U.S. National Poultry Research Center (USNPRC), Athens, Georgia or National Veterinary Services Laboratory (NVSL), Ames, Iowa.

2.2. Animals

Commercial day-of-hatch Nicholas turkey poults (*Meleagris gallopavo*) were received from a hatchery (Butterball, Goldsboro, NC (for USNPRC) or Valley of the Moon Hatchery, Osceola, IA (for NVSL)). The poults were mixed sex, unvaccinated and group housed in ABSL2 facilities for vaccination, and transferred to ABSL3E facilities for viral challenge. While in high containment facilities, poults at USNPRC were maintained in HEPA filtered isolation (ABSL3E) cabinets, whereas poults at NVSL were group housed under BSL3Ag biocontainment. All poults had *ad libidum* access to feed and water. All animal experimental procedures were conducted as approved by the respective Institutional Animal Care and Use Committees.

2.3. Preparation of vaccines

For the rgH5 vaccine, the hemagglutinin (HA) gene from A/Gyrfalcon/Washington/41088-6/2014 was *de novo* synthesized (Integrated DNA Technologies, Coralville, Iowa) with a modification of the HA cleavage site to be compatible with a H5 low pathogenic avian influenza (LPAI) virus. The synthesized gene was subcloned into a plasmid that transcribes both vRNA and mRNA from the target gene. Using the modified H5 gene and the remaining 7 genes from the Puerto Rico/8/1934 (PR8) egg adapted virus inserted in the rg plasmids, virus was rescued by transfecting all 8 plasmids into 293T cell line using Lipofectamine 2000 (Life Technologies, Gaithersburg, MD) and after incubation, the cell culture supernatant was inoculated into specific pathogen free embryonated chicken eggs. The virus was passaged several times in eggs to increase the viral titer. The virus was tested by the *in vivo* pathotyping assay and found to cause no mortality in 4 week-old chickens (Alexander, 2005). We also demonstrated that the virus did not produce plaques in Mabin-Darby canine kidney cells without the addition of trypsin, and that the cleavage site of the hemagglutinin gene sequence of the rescued virus was unchanged. The RNA particle vaccine (RP-H5) was provided by the manufacturer (Merck Animal Health, Ames, IA). The H5 gene of A/Gyrfalcon/Washington/41088-6/2014 H5N2 clade 2.3.4.4, with a modification of the HA cleavage site to a LPAI virus genotype, was constructed into the replication-deficient alphavirus particles. The rHVT vaccine encoding H5 (Vectormune® AI) was provided by the manufacturer (CEVA Animal Health, Lenexa, KS). This vaccine was constructed by inserting the HA gene of the HPAI A/swan/Hungary/4999/2006 H5N1 clade 2.2 strain into the genome of HVT FC-126 strain (rHVT-AI). The cleavage site of the HA gene used in the rHVT-AI was altered to a typical LPAI virus strain.

2.4. Experiment 1–single and double rgH5 vaccine

In experiment 1, 30 poults were vaccinated at 3 weeks-of-age with beta-propiolactone (BPL)-inactivated rgH5 virus containing 512 hemagglutinin units (HAU) per dose (0.5 mL) mixed (70/30) in Montanide ISA VG70 oil emulsion adjuvant (SEPPIC, Inc., Fairfield, NJ) and delivered subcutaneously (SQ) in the neck. Thirty poults were sham vaccinated at 3 weeks-of-age with 0.5 mL of allantoic fluid in Montanide given SQ. Twenty poults from each group were challenged at 6 weeks-of-age (3 weeks post-vaccination), as described below. The remaining 10 vaccinated poults were boosted at 6 weeks-of-age with the rgH5 vaccine delivered with 512 HAU per poult delivered in 0.5 mL in adjuvant as above. Similarly, at 6 weeks-of-age sham vaccinated poults received 0.5 mL of uninfected allantoic fluid in Montanide. Poults were bled prior to vaccination and challenge, and sera were analyzed for HAI. Poults in these groups were challenged at 9 weeks-of-age (3 weeks-post-boost), as described below.

2.5. Experiment 2–single rHVT-AI and boost rgH5

Thirty poults received rHVT-AI per manufacturer's recommendation at 1 day of age delivered SQ in the neck in 0.2 mL. Thirty sham vaccinated poults receive 0.2 mL of Marek's diluent at 1 day-of-age given SQ. Twenty poults from each group were challenged at 4 weeks-of-age

(4 weeks-post-vaccination). The remaining 10 poults in the rHVT-AI group received a boost vaccination with 512 HAU rgH5 per poult delivered in 0.5 mL in Montanide at 4 weeks-of-age. Similarly, sham vaccinated poults received 0.5 mL of allantoic fluid in Montanide at 4 weeks-of-age. Poults were bled prior to vaccination and challenge, and sera were analyzed for HAI. Poults in these groups were challenged at 7 weeks-of-age (3 weeks-post-boost).

2.6. Experiment 3–single RP-H5 vaccine

Twenty poults received RP-H5 (10^7 particles/dose) per manufacturer's recommendation at 3 weeks-of-age delivered SQ in the neck in 0.2 mL. Twenty sham vaccinated poults received 0.2 mL of vaccine diluent at 3 weeks-of-age given SQ. Poults were bled prior to vaccination and challenge, and sera were analyzed for HAI. Twenty poults from each group were challenged at 6 weeks-of-age (3 weeks-post-vaccination).

2.7. Experiment 4–single rHVT-AI and boost RP-H5

Twenty poults received rHVT-AI per manufacturer's recommendation at 1 day of age delivered SQ in the neck in 0.2 mL. Twenty sham vaccinated poults received 0.2 mL of Marek's diluent at 1 day-of-age given SQ in the neck. At 4 weeks-of-age, the rHVT-AI group was vaccinated a second time with RP-H5, and the sham vaccine poults received sterile diluent, as described in experiment 3. Poults were bled prior to vaccination and challenge, and sera were analyzed for HAI. At 7 weeks-of-age (1 week after the second vaccination), both groups of poults were challenged.

2.8. Challenge

Poults were moved into higher biocontainment facilities to allow acclimation prior to being challenged intranasally (IN) with 0.2 mL containing 3×10^6 50% embryo infectious doses (EID_{50}) of Tk/MN/15 clade 2.3.4.4 H5N2 HPAI. After challenge, poults were observed twice daily, and a record of clinical signs and number of mortalities was kept. If poults were observed to be moribund (e.g., failed to rise when the room was entered or had neurological disease), they were subsequently euthanized and reported as dead the next day for statistical purposes. Serum was collected up to 14 days post-challenge, and oropharyngeal and cloacal swabs were collected 2 and 4 days-post-challenge to quantitate virus shedding. Surviving poults were euthanized 14 days-post-challenge.

2.9. Determination of virus shedding

Oropharyngeal and cloacal swabs were collected in sterile brain heart infusion medium and kept frozen at −70 °C. Viral RNA was extracted using Trizol LS reagent (Invitrogen, Calsbad, CA) and the MagMAX AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX). Quantitative real-time RT-PCR (qRRT-PCR) was performed, as previously described (Lee and Suarez, 2004)]. Briefly, qRRT-PCR targeting the influenza M gene was conducted using AgPath-ID one-step RT-PCR Kit (Ambion) and the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Calsbad, CA). For quantification of viral shedding, a standard curve was established with viral RNA extracted from the titrated challenge virus TK/MN/15. Results were reported as mean $\log_{10}$ EID_{50}/mL ± SEM equivalents and the lower limit of detection being $10^{0.9}$ EID_{50}/mL.

2.10. Determination of serum hemagglutination inhibition (HI) activity

Blood was collected from poults via brachial vein venipuncture, and serum was isolated and stored at −20 °C before use. Serum HI activity was determined using BPL inactivated Tk/MN/15 antigen (clade 2.3.4.4), as described previously (Pedersen, 2014)]. HI titers are reported as $\log_2$ values, with 3 $\log_2$ being the minimum titer considered as positive.

2.11. Sequence analysis

Percent amino acid identity of the different H5 vaccine antigens was compared to the challenge virus H5 using CLC genomics Workbench v8.5.1 (Qiagen, Waltham, MA) with Clustal W algorithm.

2.12. Statistical analysis

Kaplan–Meier survival curves were generated with Prism 7.01 (GraphPad Co., San Diego, CA). The Mantel Cox Log-rank test was used to compare survival curves between virus challenged groups (Prism 7.01). Statistical differences in mean and standard error of the mean between HI and virus titers were analyzed using ANOVA (Prism 7.01). The Fisher Exact test was used for pair-wise comparison on frequency of virus isolation between groups (SigmaStat2.0.3, SPSS Inc., Chicago, IL). Poults with titers of 0 were assigned a value of 1 for statistical purposes. Lower case letters indicate statistical significance between compared groups. All statistical tests used $P < 0.05$ as being meaningful statistically.

3. Results

3.1. Survival of poults following lethal clade 2.3.4.4 H5N2 HPAI challenge

The rgH5 and RP-H5 vaccines tested encoded for a HA that was homologous to the clade 2.3.4.4 clade H5 challenge virus with an amino acid sequence similarity of 99% while the rHVT-AI vaccine contained a clade 2.2 H5 antigen that was 90% similar to the challenge virus (data not shown). In all 4 vaccine trials, poults were intranasally challenged with 3×10^6 EID_{50} of TK/MN/15 HPAI and monitored daily for morbidity and mortality for 14 days. In all vaccine trials, vaccination significantly ($P < 0.01$) protected poults against HPAI challenge, although differences were observed in the level of protection depending on the vaccine (Fig. 1). Complete protection from lethal HPAI challenge was demonstrated in groups receiving a single or double dose of rgH5 vaccine (Fig. 1a & b). In addition, birds receiving rHVT-AI and boosted with either rgH5 or RP-H5 were completely protected from mortality (Fig. 1d & e). No clinical disease was observed in poults that received these vaccines (data not shown). A single dose of rHVT-AI protected 75% of poults against lethal challenge, as compared to sham vaccinated poults (Fig. 1c). A single dose of RP-H5 protected 65% of the challenged poults, and some of those that died demonstrated mild neurological disease (e.g., torticollis) and diarrhea (Fig. 1f). Sham vaccinated poults in each trial displayed clinical signs including depression, lethargy, diarrhea and, in some cases, torticollis. Not all sham vaccinated poults displayed clinical signs of illness prior to dying. For experiments 1, 2, 3 and 4, all sham-vaccinated poults were dead by day 7 post-challenge, with the exception of the 2× rgH5 where delayed mortality was observed. However, the mean time to death in all sham groups was relatively constant between 3 and 6 days post-challenge.

3.2. Effect of different H5 vaccines on oral shedding of virus after lethal H5N2 HPAI challenge

The rgH5 vaccine, given in 1 or 2 doses (experiment 1), and heterologous priming with rHVT-AI and boost with rgH5 (experiment 2) significantly reduced the amount of oral shedding ($P < 0.01$) of challenge virus, as well as the number of poults positive for viral shedding ($P < 0.001$) (Table 1). A single dose of rHVT-AI significantly ($P < 0.01$) reduced the amount of oral viral shedding on day 4 post-challenge, but did not affect the number of poults shedding virus, as compared to sham vaccinated poults (Table 1). A single dose of RP-H5

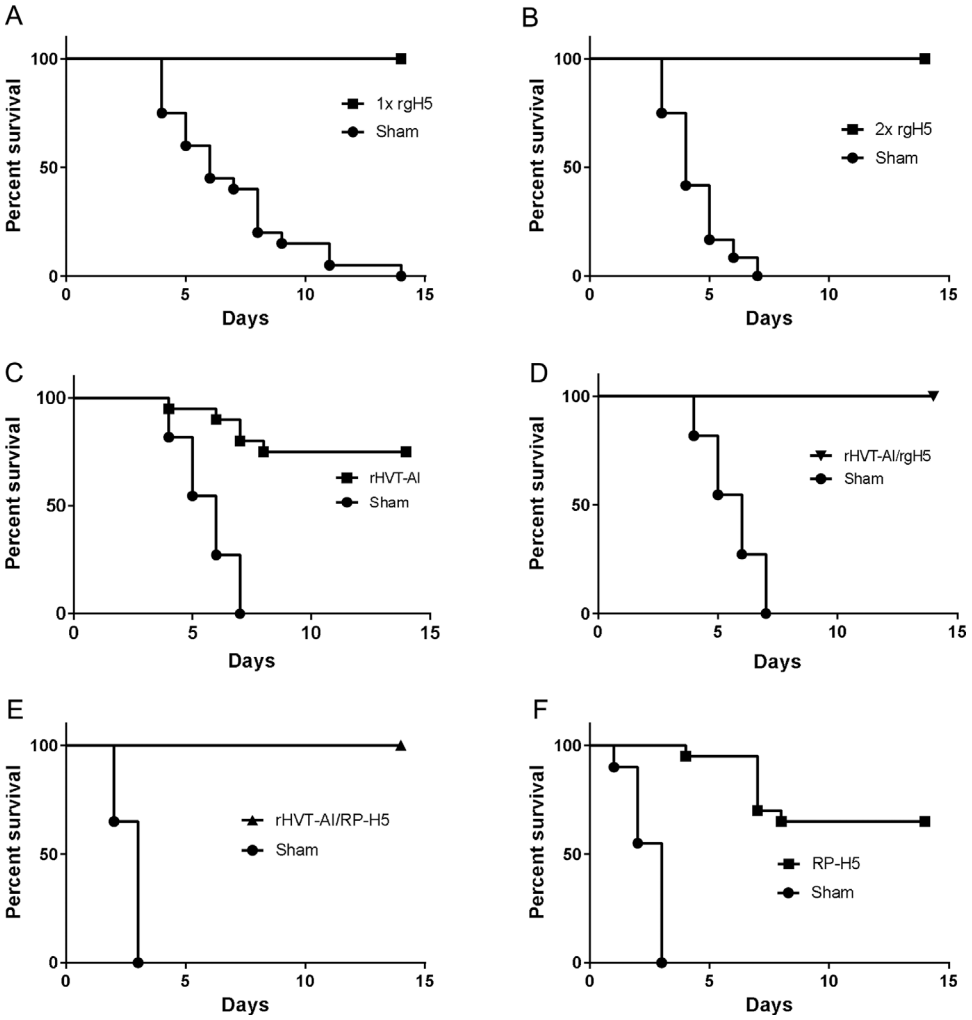

Fig. 1. Kaplan-Meier survival plots for challenge experiments 1–4. The percent survival versus day post challenge with A/turkey/Minnesota/12582/2015 H5N2 clade 2.3.4.4 HPAI for groups vaccinated with a) single dose of rgH5, b) two doses of rgH5, c) single dose of rHVT-AI, d) prime/boost with rHVT-AI and rgH5) and e) prime/boost with rHVT-AI and RP-H5, and f) single dose of RP-H5. Statistical differences in percent survival (*$P < 0.01$) were determined using Mantel-Cox log-rank test.

Table 1
Virus shedding from oropharyngeal swabs[1].

	Day 2 post-challenge		Day 4 post-challenge	
	Sham	Vaccinated	Sham	Vaccinated
Experiment 1-1x rgH5	5.31 ± 0.13 (n = 20/20)[2]	1.11 ± 0.21[3] (n = 2/20)[A]	6.43 ± 0.09 (n = 13/13)	2.24 ± 0.62[3] (n = 4/20)[A]
Experiment 1-2x rgH5	4.33 ± 0.62 (n = 7/10)	2.17 ± 0.69[3] (n = 3/10)	4.42 ± 0.69 (n = 8/9)	3.39 ± 0.21[3] (n = 2/10)[A]
Experiment 2-1x rHVT-AI	5.68 ± 0.13 (n = 20/20)	4.18 ± 0.14[3] (n = 18/20)	6.25 ± 0.09 (n = 13/13)	4.73 ± 0.14[3] (n = 19/20)
Experiment 2-rHVT-AI boost rgH5	3.79 ± 0.14 (n = 6/10)	1.12 ± 0.19[3] (n = 3/9)	4.88 ± 0.10 (n = 9/10)	1.32 ± 0.16[3] (n = 3/9)[A]
Experiment 3-1x RPH5	3.68 ± 0.15 (n = 20/20)	1.67 (n = 1/20)[A]	ND[†]	1.28 ± 0.16 (n = 4/20)
Experiment 4-rHVT-AI boost RPH5	5.16 ± 0.14 (n = 20/20)	3.3 ± 0.23[3] (n = 20/20)[A]	ND[†]	3.44 ± 0.33 (n = 19/20)

[1]Virus titer from oropharyngeal swabs, represented as mean $\log_{10}$ EID_{50}/mL ± SEM.
[2]Total number of birds positive/total number of birds swabbed .
[A]Significantly less birds shedding from sham.
[3]Significantly lower viral titers from sham.
[†]Not determined.

vaccine (experiment 3) reduced the amount of orally shed virus at day 2 post-challenge, while the number of poults shedding virus was statistically reduced. A heterologous priming with rHVT-AI and boost with RP-H5 (experiment 4) significantly reduced the amount and number of poults shedding virus on day 2 post-infection ($P < 0.001$). For experiments 3 and 4, all sham vaccinated poults were dead before day 4 post-challenge, negating the ability to statistically analyze day 4 post-challenge data for these groups.

3.3. Effect of different H5 vaccines on cloacal shedding of virus after lethal H5N2 HPAI challenge

After challenge, virus was not detected in cloacal swabs from poults receiving the rgH5 vaccine, given in 1 or 2 doses (experiment 1); or heterologous priming with rHVT-AI and boost with rgH5 (experiment 2). These vaccines significantly ($P < 0.001$) reduced the number of poults positive for cloacal viral shedding (Table 2). A single dose of rHVT-AI significantly ($P < 0.05$) reduced the amount of cloacal viral shedding at day 2 post-challenge, and significantly reduced the number of poults with cloacal virus on days 2 and 4 post-challenge, as compared to sham vaccinated poults. A single dose of RP-H5 vaccine (experiment 3) reduced the amount of orally shed virus at day 2 post-challenge and statistically reduced the number of poults shedding virus ($P < 0.001$). A heterologous priming with rHVT-AI and boost with RP-H5 (experiment 4) significantly reduced the amount and number of poults shedding cloacal virus on day 2 post-infection ($P < 0.001$). For experiments 3 and 4, all sham vaccinated poults were dead before day 4 post-challenge, negating the ability to statistically analyze day 4 data.

Table 2
Virus shedding from cloacal viral swabs[1].

	Day 2 post-challenge		Day 4 post-challenge	
	Sham	Vaccinated	Sham	Vaccinated
Experiment 1-1x rgH5	1.28 ± 0.31 (n = 6/20)[2]	Not detected (n = 0/20)[A]	3.30 ± 0.5 (n = 11/13)	Not detected (n = 0/20)[A]
Experiment 1-2x rgH5	1.71 ± 0.52 (n = 3/10)	Not detected (n = 0/10)	2.13 ± 0.22 (n = 8/9)	Not detected (n = 0/10)[A]
Experiment 2-1x rHVT-AI	4.55 ± 0.9 (n = 20/20)	3.05 ± 0.25[3] (n = 4/20)[A]	5.12 ± 1.1 (n = 13/13)	3.55 ± 1.3 (n = 9/20)[A]
Experiment 2-rHVT-AI boost rgH5	0.83 ± 0.3 (n = 5/10)	Not detected (n = 0/9)[A]	2.59 ± 1.1 (n = 10/10)	Not detected (n = 0/9)[A]
Experiment 3-1x RPH5	3.68 ± 0.15 (n = 20/20)	1.67 (n = 1/20)[A]	ND[†]	1.93 ± 1.74 (n = 4/20)
Experiment 4-rHVT-AI boost RPH5	3.93 ± 0.21 (n = 17/20)	1.94 ± 0.37[3] (n = 4/20)[A]	ND[†]	2.49 ± 0.24 (n = 18/20

[1]Virus titer from cloacal swabs, represented as mean $\log_{10}$ EID_{50}/mL ± SEM.
[2]Total number of birds positive / total number of birds swabbed.
[A]Significantly less birds shedding from sham.
[3]Significantly lower viral titers from sham.
[†]Not determined.

3.4. Effect of different H5 vaccines on pre- and post-challenge serum HI titers

All sham vaccinated poults had HI titers < 3 ($\log_2$) using clade 2.3.4.4 H5 as antigen (Table 3). In contrast, all vaccines induced elevated pre-challenge HI titers above 3.0 using the challenge antigen except the single rHVT-AI. However this was not unexpected since the HA for that vaccine was different than the 2.3.4.4 contained within the other vaccines and used in the HI test. All sham vaccinated poults died on or before day 14 post-challenge. Vaccinated survivors from the different experiments were tested for serum HI activity with the clade 2.3.4.4 H5 challenge antigen. HI titers were significantly different between vaccinated pre- and post-challenge HI titers in every vaccine group except the 2X rgH5 (Table 3).

4. Discussion

The TK/MN/15 challenge virus was isolated in April 2015 and was considered to be a representative poultry-adapted outbreak strain with slightly higher virulence and a lower turkey infectious dose50 than the reference strains isolated in December of 2014. These data are the first to demonstrate the efficacy of different H5 vaccines to protect turkey poults against lethal challenge with clade 2.3.3.4 H5 HPAI, a strain that had significant agricultural and economic impact in 2015 for U.S. turkey and chicken producers (Cima, 2015). Control of avian influenza epizootics has been accomplished by stamping out infected animals. In the case of the Mexican H5N2 epizootic in 1995, vaccination was used in conjunction with stamping out and the combination approach allowed the elimination of the HPAI virus from Mexico, but not the relayed H5N2 LPAI virus. Vaccination alone has been ineffective to control enzootic H5N1 in poultry in Egypt (Abdelwhab et al., 2016) and in other countries, and has been shown to accelerate antigenic drift of avian influenza viruses (Lee et al., 2004). The judicious use of vaccines, as a component of an AI control strategy, must be combined with additional control methods (e.g., stamping out, animal movement controls, increased biosecurity, increased surveillance), and the vaccine must be antigenically matched to the field virus, be amenable to mass-administration techniques to large numbers of poultry (Spackman and Pantin-Jackwood, 2014). Future studies are needed to continue evaluating the efficacy of *in ovo* administered H5 vaccines in poultry. The efficacy of newer vaccine technologies needs be evaluated to determine their efficacy with traditional inactivated and adjuvanted avian influenza vaccines (Rahn et al., 2015).

Table 3
Pre- and post-challenge serum HI values from HPAI H5N2 challenged poults[1].

	Pre-challenge HI		Post-challenge HI	
	Sham	Vaccinated	Sham	Vaccinated
Experiment 1-1x rgH5	< 3 (n = 20)	6.05 ± 0.15[2] (n = 20)	ND[†]	4.7 ± 0.43 (n = 20)
Experiment 1-2x rgH5	< 3 (n = 10)	6.2 ± 0.2[2] (n = 10)	ND[†]	6.4 ± 0.73 (n = 10)
Experiment 2-1x rHVT-AI	< 3 (n = 10)	1.8 ± 0.32[2] (n = 5/20)	ND[†]	6.7 ± 0.41 (n = 15/15)
Experiment 2-rHVT-AI boost rgH5	< 3 (n = 10)	4.77 ± 0.28[2] (n = 9/9)	ND[†]	6.33 ± 0.33 (n = 9/9)
Experiment 3-1x RPH5	< 3 (n = 20)	3.9 ± 0.24[2] (n = 20)	ND[†]	7.11 ± 0.33 (n = 20)
Experiment 4-rHVT-AI boost RPH5	< 3 (n = 20)	2.11 ± 0.07[2] (n = 20)	ND[†]	7.62 ± 0.24 (n = 13)

[1]Data are presented as mean $\log_2$ HI ± SEM from poults pre- and post-challenge from survivors at day 14, using clade 2.3.4.4 H5 as antigen.
[2]Statistical differences in pre-challenge versus post-challenge HI titer (*P < 0.05).
[†]Not determined.

The challenge dose of 3×10^6 EID_{50} of A/turkey/Minnesota/12582/2015 clade 2.3.4.4 H5N2 HPAI was administered to all groups, although it appeared to be more virulent in experiments 3 and 4. The mean time-to-death for these experiments was 3 days compared with 5 days for experiments 1 and 2. These differences may be explained by poults in experiments 3 and 4 being obtained from a different hatchery with different genetic stock and/or environmental differences. Because the challenge dose was rigorous, the protection afforded by these vaccines would likely be efficacious if ever applied under field conditions as a component of control mechanisms put in place during an epizootic event. The H5 vectored vaccines (e.g., rHVT-AI and RP-H5) lack neuraminidase subtype 2 (N2), and the rgH5 vaccine contained an N1, and would be considered DIVA (differentiate infected from vaccinated animals) compatible. Neuraminidase inhibition activity (Avellaneda et al., 2010) can be used to confirm DIVA and may provide confidence of the safety of imported poultry products with U.S. trade partners.

Although all H5 vaccines tested significantly increased survival and in most cases decreased viral shedding post-challenge, some vaccines and vaccine strategies may be more effective than others in controlling clade 2.3.3.4 H5N2 challenge in turkeys. The most-efficacious vaccine tested was rgH5. Groups of turkeys give either a single dose or double dose of rgH5 had pre-challenge serum HI antibody titers that would be predicted (≥40 based on chickens) to protect them from challenge. Vaccination completely prevented morbidity and mortality after challenge, and significantly reduced the amount of virus shed, as well as number of poults shedding virus. The lack of increased HI titers (e.g., anamnestic response) after challenge indicates that vaccine-induced immune responses effectively blocked viral replication. Based on these results, the rgH5 may reduce the transmission potential to naïve poults more than the other vaccines tested.

A single dose of RP-H5 (10^7 particles/dose) or rHVT-AI failed to provide 100% protection against challenge, demonstrating that these H5 vectored vaccines were not fully protective, which is similar to our previous work in chickens vaccinated with rHVT-AI and challenged with heterologous H5N1 HPAI (Kapczynski et al., 2015). It is unclear how much H5 antigen was expressed following administration of vectored rHVT-AI and RP-H5 vaccines to turkey poults, which may impact immunogenicity and protective efficacy. The significant difference in pre-challenge HI titers between poults vaccinated with rgH5 versus vectored vaccines suggests that vectored vaccines given here may produce less H5 antigen. It is possible that the amount of RP-H5 per dose was not optimized, and that increasing the particles per dose or administering a second dose may achieve higher HI titers and 100% protection. We have previously demonstrated that administering

multiple doses of replication-deficient alphavirus encoding the H5 gene protected chickens against lethal H5 HPAI challenge (Schultz-Cherry et al., 2000), and found no evidence that multiple administrations with replication-deficient alphaviruses induced anti-vector immunity (Sylte et al., 2007). The rHVT-AI was 90% similar at the amino acid level (clade 2.2) to the HA in the challenge virus, and it is likely that differences in similarity contributed to the less than 100% protection afforded by a single vaccine dose. A significant increase in post-challenge HI titer after a single dose of RP-H5 or combination of rHVT-AI and RP-H5 suggests that these vaccines failed to initially prevent viral replication, which resulted in lower survival in the RP-H5 vaccinated and challenged poults. The combination of rHVT-AI and RP-H5 must compensate for their individual lack of full protection, and induced pre-challenge HI titers below a predicted protective level. This disparity may be explained by the ability of vectored H5 vaccines to induce protective cell-mediated immunity in HPAI challenged chickens. $CD8^+$ cytotoxic T cells (CTLs) are suggested to play a role in protecting chickens and have been identified following rHVT-AI vaccination (Kapczynski et al., 2015). Although not examine in these studies, these recombinant vaccines may have induced H5-specific CTLs, which contributed to protection.

In conclusion, we report that all vaccines tested significantly protected commercial turkey poults against a lethal clade 2.3.4.4 H5N2 HPAI challenge. The rgH5 vaccine, whether administered as a single or double dose, or in combination with rHVT-AI, provided 100% protection following challenge and significantly limited viral shedding post-challenge. Other vaccines tested were less effective in controlling viral shedding post-challenge. The results from these studies led to the conditional USDA approval of commercially available vaccines for use in turkeys as a control measure for future clade 2.3.4.4 H5 HPAI epizootics.

Acknowledgements

We thank the Drs. Hank Harris and Mark Mogler (Merck Animal Health) and Drs. Yannick Gardin, John El Attrache and Kristi Moore (Ceva Animal Health) for providing the vaccines for efficacy testing. We also thank the following for their excellent technical support: Matt Inbody (ARS NADC), the Avian Viruses Section and the Proficiency Tests and Reagents group of the Diagnostic Virology Laboratory (Veterinary Services, APHIS NVSL), and Aniko Zsak (ARS USNPRC). This work was supported by USDA, ARS CRIS funds 6040-32000-062-00D. Mention of trade names or commercial products is solely for the purpose of providing specific information, and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

References

Abdelwhab, E.M., Hassan, M.K., Abdel-Moneim, A.S., Naguib, M.M., Mostafa, A., Hussein, I.T., Arafa, A., Erfan, A.M., Kilany, W.H., Agour, M.G., El-Kanawati, Z., Hussein, H.A., Selim, A.A., Kholousy, S., El-Naggar, H., El-Zoghby, E.F., Samy, A., Iqbal, M., Eid, A., Ibraheem, E.M., Pleschka, S., Veits, J., Nasef, S.A., Beer, M., Mettenleiter, T.C., Grund, C., Ali, M.M., Harder, T.C., Hafez, H.M., 2016. Introduction and enzootic of A/H5N1 in Egypt: virus evolution, pathogenicity and vaccine efficacy ten years on. Infect. Genet. Evol. 40, 80–90.

Alexander, D., 2005. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Avian Influenza. World Organisation for Animal Health, Paris, France.

Avellaneda, G., Sylte, M.J., Lee, C.W., Suarez, D.L., 2010. A heterologous neuraminidase subtype strategy for the differentiation of infected and vaccinated animals (DIVA) for avian influenza virus using an alternative neuraminidase inhibition test. Avian Dis. 54, 272–277.

Bertran, K., Swayne, D.E., Pantin-Jackwood, M.J., Kapczynski, D.R., Spackman, E., Suarez, D.L., 2016. Lack of chicken adaptation of newly emergent Eurasian H5N8 and reassortant H5N2 high pathogenicity avian influenza viruses in the U.S. is consistent with restricted poultry outbreaks in the Pacific flyway during 2014–2015. Virology 494, 190–197.

Cima, G., 2015. 47 M birds dead in flu outbreak: egg-laying hens hit hardest, followed by turkeys. J. Am. Vet. Med. Assoc. 247, 22–23.

Ip, H.S., Torchetti, M.K., Crespo, R., Kohrs, P., DeBruyn, P., Mansfield, K.G., Baszler, T., Badcoe, L., Bodenstein, B., Shearn-Bochsler, V., Killian, M.L., Pedersen, J.C., Hines, N., Gidlewski, T., DeLiberto, T., Sleeman, J.M., 2015. Novel Eurasian highly pathogenic avian influenza A H5 viruses in wild birds, Washington, USA, 2014. Emerg. Infect. Dis. 21, 886–890.

Kapczynski, D.R., Esaki, M., Dorsey, K.M., Jiang, H., Jackwood, M., Moraes, M., Gardin, Y., 2015. Vaccine protection of chickens against antigenically diverse H5 highly pathogenic avian influenza isolates with a live HVT vector vaccine expressing the influenza hemagglutinin gene derived from a clade 2.2 avian influenza virus. Vaccine 33, 1197–1205.

Kapczynski, D.R., Pantin-Jackwood, M.J., Spackman, E., Chrzastek, K., Suarez, D.L., Swayne, D.E., 2017. Homologous and heterologous antigenic matched vaccines containing different H5 hemagglutinins provide variable protection of chickens from the 2014 U.S. H5N8 and H5N2 clade 2.3.4.4 highly pathogenic avian influenza viruses. Vaccine. http://dx.doi.org/10.1016/j.vaccine.2017.04.042. pii: S0264-410X(17)30520-0, [Epub ahead of print].

Lee, C.W., Suarez, D.L., 2004. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. J. Virol. Methods 119, 151–158.

Lee, C.W., Senne, D.A., Suarez, D.L., 2004. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J. Virol. 78, 8372–8381.

Lee, D.H., Torchetti, M.K., Winker, K., Ip, H.S., Song, C.S., Swayne, D.E., 2015. Intercontinental spread of asian-origin H5N8 to north america through beringia by migratory birds. J. Virol. 89, 6521–6524.

Pedersen, J.C., 2014. Hemagglutination-inhibition assay for influenza virus subtype identification and the detection and quantitation of serum antibodies to influenza virus. Methods Mol. Biol. 1161, 11–25.

Rahn, J., Hoffmann, D., Harder, T.C., Beer, M., 2015. Vaccines against influenza A viruses in poultry and swine: status and future developments. Vaccine 33, 2414–2424.

Schultz-Cherry, S., Dybing, J.K., Davis, N.L., Williamson, C., Suarez, D.L., Johnston, R., Perdue, M.L., 2000. Influenza virus (A/HK/156/97) hemagglutinin expressed by an alphavirus replicon system protects chickens against lethal infection with Hong Kong-origin H5N1 viruses. Virology 278, 55–59.

Spackman, E., Pantin-Jackwood, M.J., 2014. Practical aspects of vaccination of poultry against avian influenza virus. Vet. J. 202, 408–415.

Stoute, S., Chin, R., Crossley, B., Gabriel Senties-Cue, C., Bickford, A., Pantin-Jackwood, M., Breitmeyer, R., Jones, A., Carnaccini, S., Shivaprasad, H.L., 2016. Highly pathogenic eurasian H5N8 avian influenza outbreaks in two commercial poultry flocks in california. Avian Dis. 60, 688–693.

Swayne, D.E., Spackman, E., Pantin-Jackwood, M., 2014. Success factors for avian influenza vaccine use in poultry and potential impact at the wild bird-agricultural interface. Ecohealth 11, 94–108.

Swayne, D.E., 2012. Impact of vaccines and vaccination on global control of avian influenza. Avian Dis. 56, 818–828.

Sylte, M.J., Hubby, B., Suarez, D.L., 2007. Influenza neuraminidase antibodies provide partial protection for chickens against high pathogenic avian influenza infection. Vaccine 25, 3763–3772.

Torchetti, M.K., Killian, M.L., Dusek, R.J., Pedersen, J.C., Hines, N., Bodenstein, B., White, C.L., Ip, H.S., 2015. Novel H5 Clade 2.3.4.4 Reassortant (H5N1) Virus from a Green-Winged Teal in Washington, USA. Genome Announc 3.

Williams, S.M., 2016. A Laboratory Manual, the Isolation, Identification and Characterization of Avian Pathogens, 6th edition. American Association of Avian Pathologists, Jacksonville, FL (pages cm p).